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Relative Contributions of *Enterococcus faecalis* OG1RF Sortase-Encoding Genes, *srtA* and *bps* (*srtC*), to Biofilm Formation and a Murine Model of Urinary Tract Infection▽†

Kelvin D. Kemp,[1]‡ Kavindra V. Singh,[1,2]‡ Sreedhar R. Nallapareddy,[1,2]‡ and Barbara E. Murray[1,2,3]*

Division of Infectious Diseases, Department of Internal Medicine,[1] Center for the Study of Emerging and Reemerging Pathogens,[2] and Department of Microbiology and Molecular Genetics,[3] University of Texas Medical School, Houston, Texas 77030

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Deletion mutants of the two sortase genes of *Enterococcus faecalis* OG1RF were constructed. *srtC* (renamed here *bps* for *b*iofilm and *p*ilus-associated *s*ortase) was previously shown to be necessary for the production of Ebp pili and important for biofilm formation and endocarditis. Here, we report that a *srtA* deletion mutant showed a small (5%) yet significant ($P = 0.037$) reduction in biofilm relative to OG1RF, while a Δ*srtA* Δ*bps* double mutant showed a much greater reduction (74% versus OG1RF and 44% versus the Δ*bps* mutant). In a murine urinary tract infection (UTI), the 50% infective doses of both the Δ*srtA* Δ*bps* and Δ*bps* mutants were ~2 $\log_{10}$ greater than that of OG1RF or the Δ*srtA* mutant. Similarly, ~2 $\log_{10}$ fewer bacteria were recovered from the kidneys after infection with the Δ*bps* mutant ($P = 0.017$) and the Δ*srtA* Δ*bps* double mutant ($P = 0.022$) compared to wild-type strain OG1RF. In a competition UTI, the Δ*bps* mutant was slightly, but not significantly, less attenuated than the Δ*srtA* Δ*bps* double mutant. Fluorescence-activated cell sorter analysis with Ebp-specific antibodies confirmed that a minority of OG1RF cells express Ebp pili on their surface in vitro and that Bps has a major role in Ebp pilus biogenesis but also indicated a function for SrtA in surface localization of the pilus subunit protein EbpA. In conclusion, deletion of *bps* had a major effect on virulence in murine UTIs, as well as biofilm; deletion of *srtA* from OG1RF had little effect on these phenotypes, but its deletion from a *bps* mutant had a pronounced effect on biofilm, suggesting that Bps and/or the proteins it anchors may compensate for the loss of some SrtA function(s).

In gram-positive bacteria, it has been known for some time that cell surface proteins can be covalently attached to peptidoglycan by a mechanism that requires sortase(s) (7, 15, 24). Sortases are extracellular transpeptidases that are found in the plasma membrane and have been shown to sort and anchor proteins that are destined for the cell surface by cleaving the conserved threonine of C-terminal LPXTG-like motifs, followed by amide bond formation between threonine and the pentaglycine cross-bridge of cell wall peptidoglycan (7, 24). Sortases can be divided into four classes, namely, A, B, C, and D, based on their structure (1). Class A sortases, which appear to be ubiquitous in many gram-positive bacteria, anchor a large number and broad range of surface proteins (7). The sortase C class of enzymes are predicted to anchor a much smaller set of substrates, and the genes coding for these are typically clustered with the substrate genes and have been shown to be involved in pilus biogenesis in addition to surface anchoring (18, 22). Genome analysis of *Enterococcus faecalis* V583 revealed the presence of two class A sortases (EF_2524 and SrtA [EF_3056]), one class C sortase (SrtC [EF_0194], renamed here Bps for *b*iofilm and *p*ilus-associated *s*ortase) (1, 16), and 41 surface proteins bearing a cell wall sorting signal motif (19). It is believed that at least some of these surface proteins are microbial surface components recognizing adhesive matrix molecules (19) that play a role in the attachment of *E. faecalis* to extracellular matrix proteins and thus are likely to be important for virulence; one example is Ace, an *E. faecalis* adhesin to collagen and laminin (11). In addition, two other sortases, named *srt-1* and *srt-2*, were reported in *E. faecalis* strain E99 containing a *bee* (*b*iofilm *e*nhancer in *e*nterococcus) locus; however, their occurrence was rare and they were found in only 2 of 40 *E. faecalis* isolates tested (23).

While analyses of sortase mutants of several gram-positive pathogens have provided evidence for their wide range of roles in bacterial physiology and pathogenesis (reviewed in reference 7), only limited information is available on the role of *E. faecalis* sortases. A study by Kristich et al. (5) demonstrated that SrtA anchors the plasmid-encoded protein Asc10 to the enterococcal cell wall to facilitate the pheromone-induced aggregation of *E. faecalis* OG1RF cells. Deletions of *bee* locus-linked *srt-1* and *srt-2* of *E. faecalis* E99 (23) and of the ubiquitous *ebp* (*e*ndocarditis and *b*iofilm-associated *p*ilus)-linked sortase C (encoded by *bps*, also referred to in earlier studies as *srtC*) of *E. faecalis* OG1RF (13) have each led to a reduction in biofilm production. It was also demonstrated that *bps*, but not *srtA*, was essential for tethering of the endocarditis-associated Ebp pili by *E. faecalis* OG1RF. However, what other proteins may be processed by Bps or SrtA and what role these sortases may play in virulence have not been reported.

The present study further explored the role of *E. faecalis* sortases by deleting independently or in combination the two

* Corresponding author. Mailing address: Division of Infectious Diseases, Department of Internal Medicine, University of Texas Medical School at Houston, 6431 Fannin Street, MSB 2.112, Houston, TX 77030. Phone: (713) 500-6745. Fax: (713) 500-6766. E-mail: bem.asst@uth.tmc.edu.

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‡ K.D.K., K.V.S., and S.R.N. contributed equally to this work.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
E. faecalis strains		
OG1RF	Laboratory strain; Rif^r Fus^r	10
CK111(pCF10)	Conjugative donor construct; Rif^s Fus^s Spe^r	5
TX5470	*bps* deletion mutant of OG1RF; OG1RF Δ*bps*[b]	13
TX5536	*srtA* deletion mutant of OG1RF; OG1RF Δ*srtA*	This study
TX5537	*srtA* and *bps* double-deletion mutant of OG1RF; OG1RF Δ*srtA* Δ*bps*	This study
E. coli strains		
EC1000	*E. coli* host strain for routine cloning of RepA-dependent plasmids	6
TX5497	EC1000(pTEX5497); Chl^r Ery^r	This study
Plasmids		
pCJK47	Plasmid for markerless exchange; carries oriT$_{pCF10}$, *lacZ*, and *P-pheS**; Ery^r	4
pTEX5497	Plasmid for *srtA* deletion with flanking regions of *srtA* cloned into pCJK47; Ery^r	This study

[a] Abbreviations: Chl, chloramphenicol; Ery, erythromycin; Fus, fusidic acid; Gen, gentamicin; Rif, rifampin; Tet, tetracycline; Spe, spectinomycin. A superscript s designates sensitivity, and a superscript r designates resistance.
[b] Previous studies referred to *bps* as *srtC*.

sortase genes (namely, *srtA* and *bps*) present in *E. faecalis* strain OG1RF. Here, we generated sortase (*srtA* with and without *bps*) deletion derivatives of OG1RF and evaluated these constructs for their phenotypic effects by using biofilm assays and a mouse urinary tract infection (UTI) model and for their effect on the surface localization of Ebp pilus subunit proteins.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are listed in Table 1. A total of 100 *E. faecalis* strains isolated over 32 years from diverse locations (the United States, Thailand, China, Argentina, Spain, Canada, Belgium, and the United Kingdom), including clinical isolates, nosocomial and community-derived fecal isolates, and animal isolates, were included. Tryptic soy broth (Difco Laboratories, Sparks, MD) containing 0.25 M glucose was used for biofilm experiments. Brain heart infusion broth (BHIB) (Difco Laboratories) and/or brain heart infusion agar (BHIA) was used for routine culturing of bacteria. BHIB containing 40% heat-inactivated horse serum (BHIS; Sigma, St. Louis, MO) was used to culture the bacteria for in vivo experiments. The antibiotics (Sigma) used in BHIA plates for the growth of OG1RF and the various mutant strains were erythromycin (10 μg/ml), fusidic acid (25 μg/ml), rifampin (100 μg/ml), and spectinomycin (1,000 μg/ml).

General techniques. Genomic DNA was extracted by the hexadecyltrimethylammonium bromide method as described previously (25). For the PCR primers used for amplification and sequencing, see Table S1 in the supplemental material. Pulsed-field gel electrophoresis was carried out as described earlier (9). Southern and colony lysate hybridizations were performed under high-stringency conditions (20) with probes labeled with the RadPrime DNA labeling system (Invitrogen).

Construction of sortase mutants. Nonpolar deletion mutants of *E. faecalis* were constructed by allelic replacement (4, 13). By crossover PCR, fragments upstream (1 kb) and downstream (900 bp) of the complete *srtA* open reading frame were amplified together to create a single 1.9-kb fragment with primers listed elsewhere (see Table S1 in the supplemental material). After ligating this fragment into pCJK47 (4), the construct was then transformed into *Escherichia coli* EC1000 (6) cells to obtain TX5497 (Table 1). After confirming the sequence, the plasmid containing the *srtA* up-down fragment (pTEX5497) was electroporated into *E. faecalis* CK111 competent cells (5). CK111 cells containing pTEX5497 were conjugated with wild-type OG1RF and TX5470 by a standard technique (12), and single-crossover integrants (OG1RF::pTEX5497 and TX5470::pTEX5497) were selected on BHIA plates which contained rifampin (100 μg/ml) with erythromycin (10 μg/ml) or spectinomycin (1,000 μg/ml). Single colonies were purified on BHIA plates with fusidic acid (25 μg/ml) with erythromycin (10 μg/ml) and subjected to the PheS* counterselection (negative selection) system with MM9YEG agar plates supplemented with 10 mM *p*-Cl-Phe (4) to select for double-crossover deletions. The deletions were confirmed as correct by PCR sequencing, hybridization, and pulsed-field gel electrophoresis procedures.

Growth curve experiment. Cultures of test bacteria grown overnight were diluted (1:20) in BHIS and grown at 37°C with gentle shaking. A reading of optical density at 600 nm (OD_{600}) was taken every hour from 0 to 12 h and then at 24 h. At intervals of 0, 4, and 6 h, CFU counts were also determined by plating serial dilutions on BHIA.

Biofilm assay. A biofilm density assay was carried out for wild-type *E. faecalis* OG1RF and its isogenic Δ*srtA*, Δ*bps*, and Δ*srtA* Δ*bps* deletion mutants according to a method previously described by Mohamed et al. (8).

UTI model. Protocols for preparation of mice, inoculum volumes, and all others stages of the experiment were the same ones previously used in our laboratory (21). Initially groups of five mice per inoculum (10^2 to 10^6 CFU) were used for each test bacterial strain (wild-type OG1RF and the Δ*srtA*, Δ*bps*, and Δ*srtA* Δ*bps* mutants), resulting in several independent monoinfection experiments. In the second set of monoinfection experiments, groups of 10 additional mice were infected with an inoculum of 10^4 CFU. The urinary bladders and kidney pairs were excised, weighed, and homogenized in 1 ml and 5 ml of saline, respectively, and dilutions were plated onto BHIA for CFU counting. The 50% infective dose (ID_{50}) of each test bacterium was determined by a previously described method (17). The detection limit of bacteria in this experiment was 10 CFU/ml of tissue homogenate. Identities of the test bacteria recovered from infected organs were confirmed by plating them on bile esculin azide agar plates (Difco Laboratories) and BHIA plates with rifampin (100 μg/ml) or by colony PCR. For the competition assay, cultures of Δ*bps* and Δ*srtA* Δ*bps* mutants were resuspended in saline solution and mixed in approximately equal (1:1) volumes based on OD_{600} readings, used to infect mice, and plated for colony counts. After 24 h, CFU were recovered from organs as previously described (21). Colony lysate preparations were probed with intragenic *bps*, *srtA* (amplified with primer sets EF3056F-EF3056R for *srtA* and EF1094F-EF1094R for *bps*), and *ace* (2) probes, and high-stringency hybridization techniques were used to determine the ratio of Δ*bps* mutant to Δ*srtA* Δ*bps* double mutant cells in the bacterial colonies recovered. Similar to the method previously described for *E. faecalis* mixed infections (13, 14), the mean virulence index of Δ*srtA* Δ*bps* mutants relative to that of Δ*bps* mutants was calculated with the following equation: Mean virulence index = Σ[(% Δ*bps* in inoculum)/(% Δ*srtA* Δ*bps* in inoculum)]/Σ[(% Δ*bps* in kidneys)/(% Δ*srtA* Δ*bps* in kidneys)].

The University of Texas Health Science Center at Houston preapproved protocol and guidelines of the Animal Welfare Committee were followed throughout the course of this study.

Polyclonal antibodies and flow cytometry. Production and purification of polyclonal antibodies against recombinant EbpA, EbpB, and EbpC proteins were described elsewhere (13). For flow cytometry analysis of surface expression of Ebp proteins, bacteria were grown in BHIS to late log phase. After being washed twice with phosphate-buffered saline (PBS), 100 μl of bacteria adjusted to an OD_{600} of 0.2 were suspended in 40 μl of newborn calf serum (Sigma) and incubated for 15 min at room temperature. After centrifugation at 10,000 × *g* for 6 min, 100 μl of 20 μg/ml preimmune or affinity-purified anti-Ebp specific

antibodies in dilution buffer (PBS containing 20% newborn calf serum and 0.1% bovine serum albumin [BSA]) was added and the solution was incubated at 4°C for 2 h. The bacteria were washed twice with 400 μl of 0.1% BSA in PBS and then added to a dilution buffer containing 100 μl of 1:100-diluted goat anti-rabbit immunoglobulin G (IgG) conjugated with $F(ab')_2$ fragment-specific *R*-phycoerythrin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and incubated at 4°C for 2 h. After cells were washed three times with 400 μl of 0.1% BSA in PBS, cells were resuspended in 500 μl of 1% paraformaldehyde in PBS and analyzed with a Coulter EPICS XL AB6064 flow cytometer (Beckman Coulter, Fullerton, CA) and System II software.

Statistics. Differences in OD_{570} readings in biofilm experiments were evaluated with the Kruskal-Wallis test (analysis of variance [ANOVA]) and, in some cases, also by the Mann-Whitney test. Differences in the $\log_{10}$ CFU of bacteria recovered from organs in monoinfections with inocula of 10^4 CFU were evaluated by the unpaired *t* test. Differences between the total numbers of infected kidney pairs/bladder (10^2 to 10^6 CFU inoculum groups combined) were evaluated by Fisher's exact test. The percentages of the Δ*bps* mutant in the inoculum versus the percentages of the Δ*bps* mutant in the kidneys of individual mice infected with the Δ*bps* and Δ*srtA* Δ*bps* mutants in the competition assay were analyzed for significance by the paired *t* test.

RESULTS AND DISCUSSION

An estimated 8 million doctor visits in the United States are linked with UTIs each year (3). Although the significance of *E. faecalis* in causing infections associated with the kidney and bladder is well documented (accounting for 2 to 12% of outpatient UTIs and 7 to 16% of inpatient UTIs) (26), little is known about the factors used by *E. faecalis* to infect this site. To help develop therapeutic or preventive measures against *E. faecalis* UTIs, it is important to identify *E. faecalis* surface antigens which function as adhesins to urinary tract tissues. Cell wall-associated proteins of *E. faecalis* are the focus of our ongoing research because of their predicted central role in adhesion and, presumably, colonization of tissues (e.g., heart valves and kidneys) and infections (e.g., endocarditis and UTIs). Since these surface proteins are thought to utilize the sortase-mediated cell wall anchoring mechanism, we here constructed and analyzed different sortase mutants to indirectly assess the global effect of surface-associated proteins in biofilm formation and pathogenicity of *E. faecalis* in UTIs. Conceptually, we consider the sortases to be fitness factors, rather than virulence factors, since the impact of sortase deletions is presumably the result of defective localization of a number of cell wall-associated proteins, including virulence factors.

Sortase-encoding genes of *E. faecalis* strains. Of the three sortase genes (*srtA*, EF_2524, and *bps*) previously identified in strain V583 (16), EF_2524 (which is part of an integrated plasmid remnant region [EF_2512 to EF_2542]) of V583 (16) was not present in seven of nine *E. faecalis* strains tested, including OG1RF (1, 13). Since our previous study found that *bps* is ubiquitous in *E. faecalis* (13), here we tested for the presence of *srtA* in 100 diverse *E. faecalis* strains by colony hybridization and found that this gene was also present in all of the isolates tested. The ubiquitous distribution of these two genes in diverse *E. faecalis* strains suggests that they are part of the core genome and have a role in *E. faecalis* biology. We next analyzed the almost completed genomic sequence of *E. faecalis* strain OG1RF (10) generated by our collaboration with the human genome sequencing center at the Baylor College of Medicine (available at http://www.hgsc.bcm.tmc.edu/projects/microbial/microbial-detail.xsp?project_id=111) and confirmed that OG1RF (which failed to hybridize with EF_2524) contains only the two ubiquitous sortase genes *srtA* and *bps*. In order to evaluate the independent roles of these sortase genes, we generated sortase (*srtA* and/or *bps*) deletion derivatives of strain OG1RF.

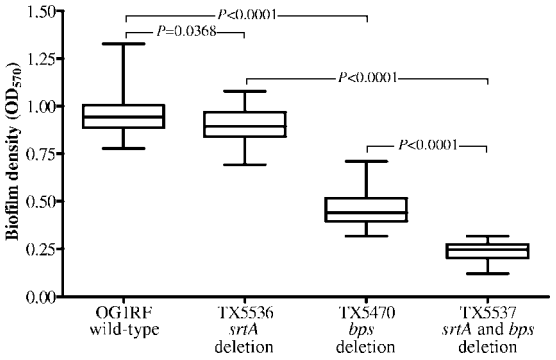

FIG. 1. Comparison of biofilm production of wild-type OG1RF and the TX5536 (Δ*srtA*), TX5470 (Δ*bps*), and TX5537 (Δ*srtA* Δ*bps*) mutants. Median values and interquartile ranges are shown. Multiple comparison of median OD_{570}s from biofilm assays of wild-type and mutant cells by Kruskal-Wallis test (ANOVA) with Dunn's post-hoc modification showed a highly significant difference ($P < 0.0001$). Individual median OD_{570}s from biofilm assays of wild-type and mutant cells were compared by Mann-Whitney test and are shown above the whiskers.

Deletion of *E. faecalis srtA* from *OG1RF* and from its *bps* deletion mutant, TX5470. Sequencing of OG1RF Δ*srtA* (TX5536) and OG1RF Δ*srtA* Δ*bps* (TX5537) confirmed the deletion of the complete *srtA* open reading frame, which encodes 244 amino acids (data not shown). There were no obvious differences in the growth patterns of wild-type *E. faecalis* OG1RF and its isogenic sortase mutants (Δ*srtA*, Δ*bps*, and Δ*srtA* Δ*bps*), suggesting that the sortases are not essential for in vitro growth of *E. faecalis*.

Contribution of the *srtA* and *bps* genes to biofilm formation. All of the sortase mutant derivatives of *E. faecalis* OG1RF showed decreased biofilm formation ($P < 0.0001$ by Kruskal-Wallis test), with reductions ranging from 5% (Δ*srtA* mutant; median OD_{570} = 0.893; interquartile range [IQR], 0.841 to 0.968) to 74% (Δ*srtA* Δ*bps* double mutant; median OD_{570} = 0.247; IQR, 0.204 to 0.274) relative to wild-type *E. faecalis* OG1RF (median OD_{570} = 0.943; IQR, 0.887 to 1.004) (Fig. 1). The decrease for the Δ*srtA* mutant was small and not significant ($P > 0.05$) by post-hoc test of Kruskal-Wallis ANOVA testing compared to OG1RF, although it was significant by the Mann Whitney test (Fig. 1) ($P = 0.037$), and observed in repeated experiments. The decrease seen with the Δ*srtA* Δ*bps* double mutant (see above) was much more substantial, with the median OD_{570} of Δ*srtA* Δ*bps* 44% less than that of Δ*bps* (median OD_{570} = 0.441; IQR, 0.396 to 0.516; $P < 0.001$); the results for Δ*bps* are consistent with those previously reported (13). This surprising and pronounced decrease seen with the Δ*srtA* Δ*bps* double mutant versus the Δ*bps* mutant suggests the possibilities that (i) Bps may compensate for the loss of SrtA from OG1RF and that (ii) in the absence of Bps, SrtA performs some function that is important for biofilm formation.

Effect of *srtA* and *bps* gene deletions in a murine UTI model. In a recent study, we established a murine UTI model with OG1RF grown in BHIS and showed that Ebp pili are important for this strain's ability to cause infection of murine kidneys

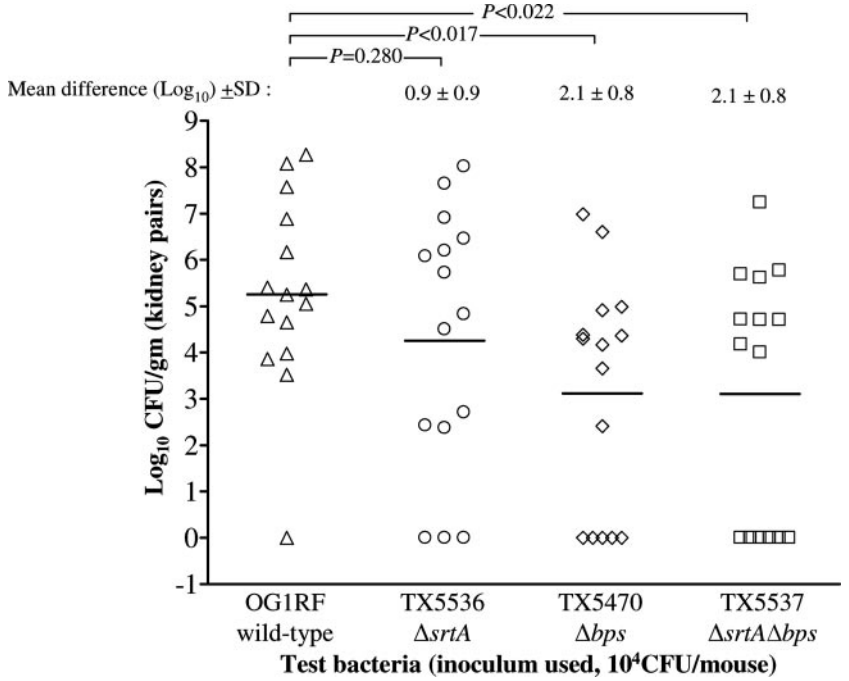

FIG. 2. Monoinfection with wild-type *E. faecalis* OG1RF and its isogenic mutants. Data are from 15 mice infected with 10^4 CFU; results are expressed as $\log_{10}$ CFU per gram from kidney homogenates 48 h after transurethral challenge. The $\log_{10}$ CFU from both kidneys were combined and averaged. A value of 1 was assigned to those kidneys with 0 CFU. Triangles represent wild-type *E. faecalis* OG1RF, circles represent OG1RF Δ*srtA*, diamonds represent OG1RF Δ*bps*, and squares represent OG1RF Δ*srtA* Δ*bps*. Horizontal bars represent the geometric mean. The mean difference in CFU counts of sortase mutants versus OG1RF is given as the $\log_{10}$ ± the standard deviation (SD). Differences in the $\log_{10}$ CFU of OG1RF versus the Δ*srtA*, Δ*bps*, and Δ*srtA* Δ*bps* mutants recovered from organs were evaluated by the unpaired *t* test.

(21). Here, to test the effects of different sortase deletion mutant derivatives of OG1RF, mice were infected as previously described (21) via intraurethral catheterization with a range of CFU. The ID_{50}s derived from monoinfection experiments showed that both the Δ*srtA* Δ*bps* ($ID_{50} = 1.5 \times 10^4$) and Δ*bps* ($ID_{50} = 3.6 \times 10^4$) mutants required ~2 $\log_{10}$ more cells to infect 50% of the mice than did wild-type *E. faecalis* OG1RF ($ID_{50} = 1.1 \times 10^2$) or Δ*srtA* ($ID_{50} = 1.4 \times 10^2$), indicating a substantial role for Bps compared to SrtA in UTI infections. Furthermore, the comparable (~2 log) difference between the ID_{50}s of the *bps* mutant and that of strain OG1RF versus those of the previously reported *ebp* mutant and strain OG1RF (21) suggests that the effect seen after *bps* deletion is likely due to the resulting inability to form Ebp pili (13). For the total number of mouse kidneys and urinary bladders infected by the mutants compared to the number infected by wild-type *E. faecalis* OG1RF, see Table S2 in the supplemental material. The percentage of kidneys of mice infected summed across all inocula was 91% for wild-type OG1RF, 80% for the Δ*srtA* mutant ($P = 0.2650$), 63% for the Δ*bps* mutant ($P = 0.0048$), and 51% for the Δ*srtA* Δ*bps* double mutant ($P < 0.0001$). Although fewer bladders than kidneys were infected, the differences between individual strains were similar to those of kidney infections (see Table S2 in the supplemental material); the percentage of bladders of mice infected summed across all inocula was 78% for wild-type OG1RF, 76% for the Δ*srtA* mutant ($P = 1.0$), 51% for the Δ*bps* mutant ($P = 0.0177$), and 37% for the Δ*srtA* Δ*bps* double mutant ($P = 0.0005$).

The $\log_{10}$ CFU recovered from the kidney pairs ($n = 15$) of mice infected with wild-type *E. faecalis* OG1RF and its isogenic mutants at a 10^4 CFU inoculum are shown in Fig. 2. A comparison of the mean $\log_{10}$ CFU of bacteria recovered from the kidneys showed that the differences between OG1RF and the Δ*bps* mutant (2.1 ± 0.8 $\log_{10}$) and between OG1RF and the Δ*srtA* Δ*bps* double mutant (2.1 ± 0.8 $\log_{10}$) were statistically significant ($P = 0.017$ and $P = 0.022$, respectively), while the difference between OG1RF and the Δ*srtA* mutant (0.9 ± 0.9, $P = 0.280$) was not.

Because the results shown above for the percentage of mice infected, although not significant by Fisher's test (see Table S2 in the supplemental material), suggested that the Δ*srtA* Δ*bps* double mutant may be more attenuated than the Δ*bps* mutant, we next used a competition assay with an approximately equal mixture of Δ*bps* and Δ*srtA* Δ*bps* mutant cells of two different inocula. For 17 animals, the percentage of Δ*bps* mutant cells in the total inoculum was 42% to 43%. At autopsy, the percentages of Δ*bps* mutant cells in the total number of CFU recovered from kidneys were in the range of 52 to 63% for five animals and 92%, 46%, and 23% for one animal each. Nine animals were not infected. The mean percentage of Δ*bps* mutant cells in the total number of CFU of bacteria recovered from kidneys was 55.1%, demonstrating a slight outnumbering by the Δ*bps* mutant; however, the paired *t* test showed no significant difference between these mutants ($P = 0.1097$). The mean virulence index (13, 14) of the Δ*srtA* Δ*bps* double mutant relative to the Δ*bps* mutant in kidneys was 0.605, indicating that SrtA plays only a minor role in UTI or that Bps can compensate for the SrtA loss. Taken together, our *bps* mutant

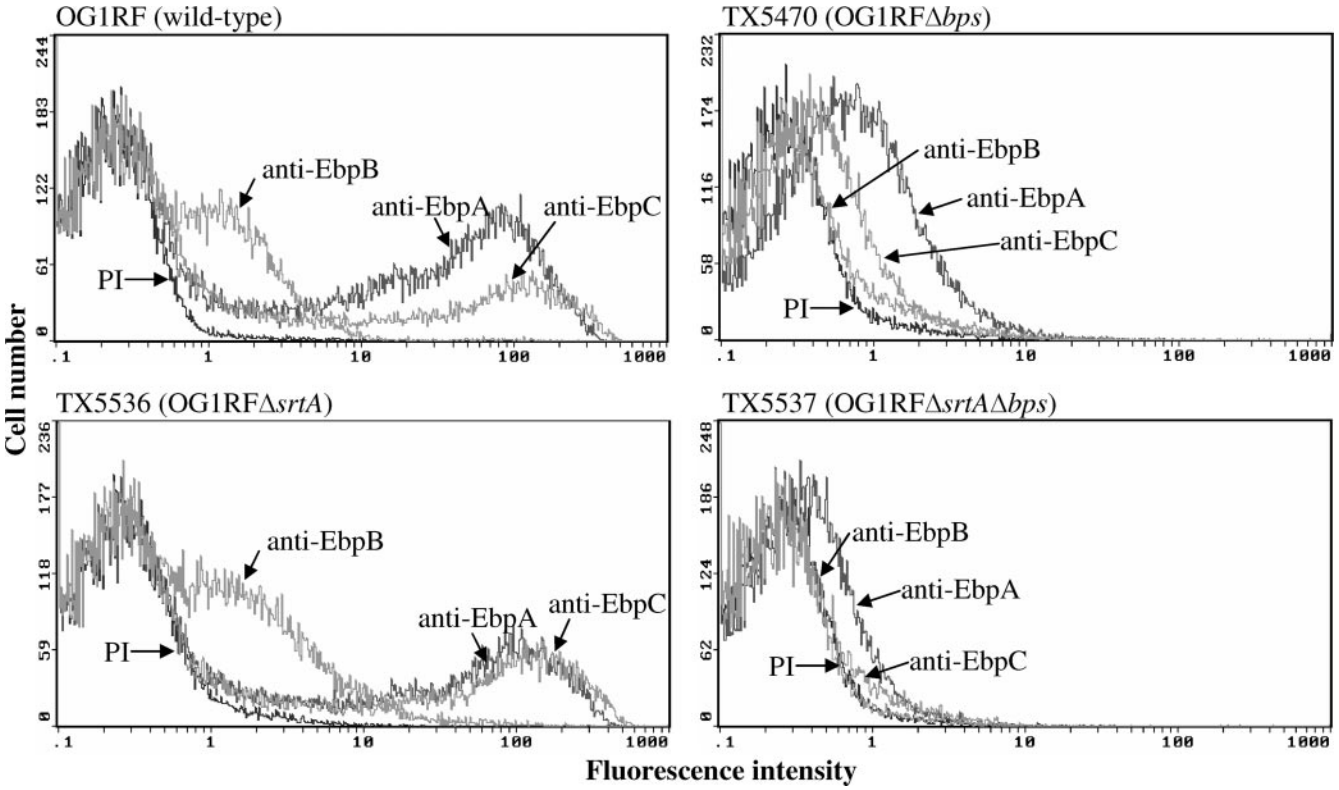

FIG. 3. Flow cytometry analysis of surface expression of Ebp pilus proteins in wild-type *E. faecalis* OG1RF and its isogenic mutants. Cells were incubated with either control preimmune (PI) IgGs or anti-Ebp specific IgGs, followed by incubation with a $F(ab')_2$ fragment of goat anti-rabbit IgGs (heavy plus light chains) conjugated to *R*-phycoerythrin. Bacteria were analyzed by flow cytometry by using side scatter as the threshold of detection. Specific binding by anti-Ebp antibodies is indicated as log fluorescence intensity on the *x* axis, and each histogram represent 50,000 bacteria.

data corroborate the previously demonstrated role of Ebp pili (21) in murine kidney infections and the competition assay results suggest little to no additional role for SrtA in this model. This is in contrast to the role of SrtA in other gram-positive organisms, such as *Staphylococcus aureus* and *Listeria monocytogenes*, where SrtA plays a much larger role in virulence (7). However, it is possible that *srtA* of *E. faecalis* contributes more significantly to the UTI disease process in humans compared to mice or it may be more important for other infection processes such as endocarditis.

Role of sortases in surface localization and polymerization of Ebp pili. By Western blotting, we have previously shown that *bps*, but not *srtA*, is needed for polymerization of Ebp pili (13) and immunogold electron microscopy estimated that few wild-type cells (<2% of cells grown in BHIB and <20% of cells grown in tryptic soy broth–0.25 M glucose) produce Ebp pili in vitro (13). Here, we measured the surface-localized Ebp proteins of wild-type OG1RF and sortase mutants after growth in BHIS (the growth condition that enhanced levels of high-molecular-weight Ebp bands in Western blot assays) (13) by fluorescence-activated cell sorting analysis. While anti-Ace antibodies (used as a positive control) bound 98% of wild-type OG1RF grown at 46°C (11) (data not shown), the percentages of EbpA-, EbpB-, and EbpC-expressing cells in BHIS cultures of strain OG1RF were 34%, 20%, and 24%, respectively (Fig. 3) and the mean fluorescence intensities of EbpA, EbpB, and EbpC were 29.8, 2.4, and 27.1, respectively. The relatively low intensity of EbpB observed in flow cytometry analysis seemed to contradict the immunoblotting data from our earlier study (13), which showed that the EbpB protein is part of the polymeric structure. One possible explanation for these results is that EbpB may be relatively hidden beneath the surface of the Ebp pilus structure and thus not accessible to antibodies in a flow cytometry analysis, as seen with group B streptococci (22). The percentages of Δ*bps* mutant cells with surface-localized EbpA, EbpB, and EbpC were 28%, 8%, and 10%, but with much lower mean fluorescence intensities (2.1, 3.0, and 2.0, respectively) (Fig. 3). This markedly reduced fluorescence intensity of Ebp proteins in the Δ*bps* mutant is consistent with the role of Bps in pilus polymerization, and this observation is consistent with loss of Ebp high-molecular-weight multimers in Western blot assays of the Δ*bps* mutant (13). Since our earlier EbpA surface localization studies by immunogold electron microscopy (13) showed staining of both the cell surface and the pili of wild-type OG1RF, the persistence of a relatively high percentage of EbpA-positive Δ*bps* mutant cells (28%), but with a greatly reduced mean fluorescence intensity (29.8 to 2.1), suggests that the EbpA subunit protein is still located on the cell surface of the *bps* mutant, presumably in the monomeric form. Unlike the Δ*bps* mutant, the Δ*srtA* mutant (TX5536) showed an expression pattern similar to that of wild-type OG1RF, albeit with higher mean fluorescence intensities (49.7,

3.3, and 34.3 for EbpA, EbpB, and EbpC, respectively) and some difference in the percentage of cells, with a small decrease for EbpA (34 versus 27%) and small increases for EbpB (20 versus 26%) and EbpC (24 versus 26%) (Fig. 3). These moderately increased Ebp intensities with the *srtA* mutant, implying enhanced pilus production compared to that of the wild type, suggest that the presence of SrtA somehow results in interference with the action of Bps for polymerization. The mutant that lacked both sortases (TX5537) showed the least surface localization (6%, 3%, and 5% of cells with mean fluorescence intensities of 1.8, 2.4, and 2.4 for EbpA, EbpB, and EbpC, respectively) (Fig. 3). The decrease in the percentage of EbpA-stained cells of the Δ*srtA* Δ*bps* double mutant (6%) compared to the Δ*bps* mutant (28%) indicates that SrtA may be involved in the surface anchoring of EbpA, at least in the absence of Bps; an additional decrease was also seen to a minor degree with both EbpB and EbpC. Taken together, these surface analysis studies indicate that both sortases are important for Ebp component surface localization in OG1RF, with Bps being the major one for pilus biogenesis, as well as for ascending murine UTI.

In summary, from this study and the previous work from our laboratory (13, 21), it appears likely that Bps, presumably via Bps-anchored surface proteins, plays an important role in both in vitro biofilm formation and in vivo murine kidney infections under the conditions tested. The results from this study also provide evidence for an additional role of SrtA or SrtA-anchored surface proteins in biofilm, particularly in the absence of Bps, but a minor role in UTI. Flow cytometry studies have shown that both sortases are important for Ebp pilus component surface localization in OG1RF, with the class C sortase Bps being the major one for pilus assembly. The ubiquitous presence of both sortase genes in *E. faecalis* isolates increases the likelihood that sortases in general, and Bps in particular, could be a target for disease prevention.

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